

ACUTE ASSAY WITH FATHEAD MINNOWS (*Pimephales promelas*)

1. TEST OBJECTIVE

Definitive Assay - To assess the toxicity of a test material to *Pimephales promelas* and determine the LC50 or EC50 using mortality and/or immobilization, respectively, as the test endpoint(s).

Screening Assay - To assess the toxicity of a test material to *Pimephales promelas* at a single test concentration (e.g., 100 percent effluent or Instream Waste Concentration).

2. TEST ARTICLE

2.1 Description/Identification

Unless otherwise specified, the test material is supplied by the client. Adequate chemical specifications with special reference to hazardous properties and storage conditions are also supplied by the client.

2.2 Methods of Synthesis

In most cases, the test article is an effluent sample. Information on the methods of synthesis, stability, and composition or other characteristics which define the test article are on file with the client.

3. EXPERIMENTAL DESIGN

3.1 Test Organisms

3.1.1 Species

The test species is the fathead minnow, *Pimephales promelas*.

3.1.2 Source

P. promelas used for toxicity tests are usually obtained from stock cultures maintained at EA's Culture Facility. However, organisms may be obtained from a scientific organism vendor, if necessary.

3.1.3 Culturing and Holding Conditions

P. promelas stock are maintained at $20 \pm 2^\circ\text{C}$ and a 16-hour light, 8-hour dark photoperiod cycle in an environmentally controlled laboratory. Test organisms are maintained in 18.9-L or 30-L all glass aquaria in a recirculating system of dechlorinated municipal tap water. If organisms are obtained from a scientific vendor, the fish, upon receipt, are transferred to static recirculating holding tanks of an appropriate size containing dechlorinated tap water at the temperature ($\pm 2^\circ\text{C}$) of the water in which the organisms were shipped. All fish stocks are examined regularly. Dead fish, or those displaying abnormal swimming behavior, discoloration, or pronounced lethargy are removed as observed, and recorded on appropriate log sheets. Fish stocks are fed a commercial fish food or *Artemia* nauplii a minimum of once daily. Certain regulatory or project specific objectives may require organism acclimation to the dilution water when it is different from the holding/culture water.

3.1.4 Age of Test Organisms at Test Initiation

The age of the test organisms is dependent on the objectives of the study and the specific guidelines being followed, but in general, fathead minnows used for acute toxicity testing are 1-14 days old (with a 24-hour range in age) upon test initiation.

3.2 Dilution Water

The source of dechlorinated tap water is the City of Baltimore municipal water system. Upon entry to the laboratory, the water passes through a high-capacity, activated-carbon filtration system to remove chlorine and other possible organic contaminants. This water source has proven safe for aquatic organism toxicity testing at EA, as evidenced by maintenance of multigeneration *Daphnia* sp. and fathead minnow cultures with no evident loss of fecundity. Reconstituted fresh water or other dilution water may be used depending on study requirements.

3.3 Test Concentration Series

The test concentration series consists of a minimum of five dilutions (e.g., 6.25, 12.5, 25, 50, and 100 percent effluent plus a control) and may be determined from a prior screening of the test material. Rangefinding assays utilize more widely spaced test concentrations and a control. Ambient water or effluent samples may also be evaluated as single concentrations and compared to a control.

3.4 Test Concentration Preparation

Test concentrations are prepared with Class A glassware.

3.5 Test Vessels and Test Volume

Test vessels are 1-L beakers; the final test volume is 250 ml. Other test vessels/volumes may be used depending on the study's requirements.

3.6 Test Organism Number

Tests are conducted using two replicates per concentration, with ten organisms per container. Fish are randomly assigned to each replicate test container. More replicates can be added, if appropriate.

3.7 Test Environment

The test vessels are maintained at $20 \pm 1^\circ\text{C}$ or $25 \pm 1^\circ\text{C}$ (unless a different project-specific temperature is required) in an environmentally controlled laboratory with a 16-hour light, 8-hour dark photoperiod. Temperature within the environmental room is monitored continuously with temperature recorders.

3.8 Analysis of Test Concentrations for Test Article

If required, test solutions may be analyzed for verification of chemical concentrations. The analytical method and number of analyses are determined after consultation with the client. When chemical analyses are necessary, both nominal and actual measured test concentrations are reported.

3.9 Test Observations

Each test day, test organisms are observed to record the number of surviving organisms. Dead organisms are removed when observed. The study terminates after completion of the observation period (24 to 96 hours). The study may be extended, however, at the request of the client.

Each effluent or receiving water sample received is analyzed for temperature, conductivity, alkalinity, hardness, and total residual chlorine. Aliquots of effluent and receiving water may be gently aerated (100 bubbles/min) prior to test initiation if dissolved oxygen is less than 4 mg/L or greater than 100 percent saturation. After test initiation, if the dissolved oxygen in any test chamber is less than 4 mg/L, all test chambers are gently aerated or other corrective action is taken. Measurements of water quality taken daily include dissolved oxygen, pH, temperature, and conductivity from a minimum of one replicate of every concentration. Analytical determinations are conducted according to APHA et al. (1995) and US EPA (1979).

At the end of the test period, a minimum of 30 surviving fish are removed from the test vessels, rinsed and placed in labeled containers, and frozen for later measurements. The fish are subsequently thawed, measured to the nearest mm (standard length), patted dry, and weighed to the nearest 0.01 g. The average length and weight of the test fish are reported, along with their respective ranges and standard deviations. For fish between 1-14 days old and within a 24-hour age range, weights and lengths usually will not be measured.

3.10 Solution Renewal (When Applicable)

When static-renewal testing is required, the test solution is renewed daily. New solutions are prepared on the day of renewal. After the new solutions have reached test temperature and water quality measurements (temperature, pH, dissolved oxygen, and conductivity) are completed, the solution renewal may be performed by transferring the organisms from one test chamber to another or by replacing the test solution. If the test solution replacement method is used, caution must be given not to stress the test organisms while the test chamber is siphoned. Usually 75 percent of the old solution is removed and replaced; however, the amount is dependent on the size of the test organisms.

3.11 Data Analysis

The LC50 and/or EC50 values and associated statistics are calculated using the probit, moving average, and binomial methods as described by Stephan (1977). Depending on the nature of the data, other methods may be used including the Trimmed Spearman-Kärber Method, the probit approximation method of Litchfield and Wilcoxon (1949), SAS probit analysis (SAS Institute 1985), or graphical interpolation using the log concentration vs. percent mortality and/or percent affected as described by APHA et al. (1995). The methods used are specified in the final report.

3.12 Test Applicability

An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests.

4. FINAL REPORT

The final report is prepared to contain, at a minimum, the following information:

- ☐ Objectives and procedures stated in the approved protocol, including any changes made to the original protocol
- ☐ Identity of the test article(s) by name or code number and their strength (i.e.,

quality/purity), and a description of any pretreatment

- ☐ Source of the dilution water, its chemical characteristics, and a description of any pretreatment
- ☐ Test concentration series used and duration of the assay
- ☐ Mean standard lengths and wet weights of test fish, the respective standard deviations, and approximate biomass loadings (g fish/L of test solution), if applicable
- ☐ Water quality characteristics (pH, dissolved oxygen, temperature, etc.) of dilution water and selected test concentrations during testing
- ☐ Any unforeseen circumstances that may have affected the quality or integrity of the study
- ☐ Signature of the project manager, senior technical reviewer, and quality control officer authorizing release of the report
- ☐ Location of all archived data and the original copy of the final report at EA

Items of data to be included in the report consist of experimental design and test performance; effects on general appearance of test organisms (if applicable); morbidity and mortality; presentation of water quality characteristics, and survival data.

5. QUALITY ASSURANCE

5.1 Amendments to Protocol

Amendments to the authorized protocol established by EA or by the client are made only after proper authorization. Such authorization is achieved by completion of the Protocol Amendment Form by EA after consultation with the client.

5.2 Standard Operating Procedures

Unless otherwise specified, all procedures given in the protocol are subject to detailed Standard Operating Procedures (SOPs) which are contained in the SOP manuals of the participating departments. These SOPs and protocols generally follow the types of requirements outlined in the U.S. EPA's Good Laboratory Practice Standards (GLPs) (US EPA 1989).

5.3 Reference Toxicant

A reference toxicant test, utilizing sodium dodecyl sulfate (SDS), cadmium chloride, or another appropriate chemical is used as an internal quality check of the sensitivity of the test organisms. Testing is conducted at least once monthly on organisms which are cultured in-house, and on each population of organisms purchased for testing from an outside source if reference toxicant data are not available from the supplier on the acquired lot. The results of each test are compared with historical, species-specific toxicological information from reference toxicant tests performed at EA, to determine if the results are within acceptable limits. Limits are established using the control charts outlined in US EPA (1993).

5.4 Quality Assurance Evaluation

Studies conducted under this protocol may be subject to internal audit by EA's Quality Assurance Unit. A quality control officer is responsible for monitoring each study to assure the client that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with EA's QC program and, if applicable, EPA's GLPs.

5.5 Inspection by Regulatory Authorities

In the event of an inspection of EA by an outside authority during the course of the study, the client whose study is being inspected will be consulted before examiners are permitted access to any of the project records or the experimental areas.

5.6 Archives

Copies of project-specific records shall be transferred to the client promptly after the project is completed or as negotiated and budgeted. Original primary data are retained at EA for 5 years. Primary data include chain-of-custody records, laboratory data sheets, records, memoranda, notes, photographs, microfilm, and computer printouts that are a result of the original observations and activities of the study and which are necessary for the reconstruction and evaluation of the study report.

5.7 Location

Studies are conducted at the Ecotoxicology Laboratory of EA Engineering, Science, and Technology, Inc. at the Loveton Office in Sparks, Maryland.

6. SPECIFICATIONS OF THE FATHEAD MINNOW ACUTE TOXICITY TEST

6.1 Basic References

- American Public Health Association (APHA) American Water Works Association, Water Environment Federation. 1995. Standard Methods for Examination of Water and Wastewater, 19th or most recent version. APHA, Washington, D.C.
- American Society for Testing and Materials (ASTM). 1991. Standard Practice for Conducting Acute Tests with Fishes, Macroinvertebrates, and Amphibians. ASTM Designation: E729-80, Philadelphia, Pennsylvania.
- EA. 1996. Quality Control and Standard Operating Procedures Manual for EA's Ecotoxicology Laboratory. Fifth Revision. EA Manual ATS-102. Internal document prepared by EA's Ecotoxicology Laboratory, EA Engineering, Science, and Technology, Inc., Sparks, Maryland.
- Litchfield, J.T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharm. Exp. Ther. 96:99-113.
- SAS Institute Inc. 1985. SAS User's Guide: Basics, Version 5 Edition. Cary, NC: SAS Institute Inc. 1290 pp.
- Stephan, C.E. 1977. Methods for calculating an LC50, in Aquatic Toxicology and Hazard Evaluation (F.L. Mayer and J.L. Hamelink, eds.), pp. 65-84. ASTM STP 634. American Society for Testing and Materials, Philadelphia, Pennsylvania.
- US EPA. 1979. Methods for Chemical Analysis of Water and Wastes. EPA/600/4-79/020. U.S. Environmental Protection Agency, Washington, D.C.
- US EPA. 1989. Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards. Title 40 CFR Part 792. Fed. Regist. 54(158): 34034-34074.
- US EPA. 1991. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.
- US EPA. 1993. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027F. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

6.2 Test Specifications

Test organism:	Fathead minnow (<i>Pimephales promelas</i>)
Temperature:	20±1 °C or 25±1 °C
Organism age:	Dependent on test guidelines being followed, but in general, 1-14 days with a 24-hour range in age
Organism length:	The largest fish is not more than 50 percent longer than the smallest fish
Loading ratio:	In general, test containers may not contain greater than 0.65 g/L biomass
Aeration:	None, unless dissolved oxygen falls below 4 mg/L
Light quality:	Wide-spectrum fluorescent light
Light intensity:	50-100 f.c.
Photoperiod:	16-hour light, 8-hour dark
Dilution water:	Dechlorinated municipal tap water, reconstituted fresh water, or appropriate receiving water
Test containers:	1-L beakers
Test volume:	250 ml per replicate
No. of concentrations:	Definitive assay - Minimum of five test concentrations and a control Screening assay - Single test concentration and a control
No. of replicates:	2
No. organisms per container:	10 (if loading is exceeded, more replicates or larger

test containers may be used)

Feeding regime: Animals are not fed during tests unless fish are too small to survive 96 hours without feeding.

Test type and duration: 24- to 96-hour acute toxicity test

Endpoints: Mortality and/or Immobilization

Immobilization--defined as cessation of movement except for minor activity of appendages

Mortality--defined as cessation of opercular movement and inability to respond to external stimuli (gentle prodding with glass rod)

Test acceptability: 90 percent or greater survival in the control solution